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SHORT COMMUNICATION

Single nucleotide polymorphisms from cytochrome b gene as a useful protocol in forensic genetics against the illegal hunting of manatees: *Trichechus manatus*, *Trichechus inunguis*, *Trichechus senegalensis*, and *Dugong dugon* (Eutheria: Sirenia)

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ABSTRACT. The identification of mitochondrial DNA polymorphisms is one of the most efficient methods for species differentiation. Genotyping of molecular markers using PCR/RFLP is a reliable, sensitive and inexpensive method for the detection of species specific mutations. The major causes of decline in Sirenia populations are accidental and intentional catches, collisions with boats and habitat loss. The goal of the present study was to identify, *in silico*, nucleotide mutations in the cytochrome b gene that can be used for the future development of forensic tools capable of using small tissue fragments to discriminate manatee meat from domesticated species meat commonly used as food sources (bovine, ovine, caprine and swine). DNA sequence alignments revealed two polymorphic sites distinguishing the manatee species from domestic species. The present study reinforced the power of cytochrome polymorphisms as powerful markers for species identification, which may be particularly useful for identifying vulnerable/endangered species. The data provided herein also suggest such mtDNA markers as important conservation tools for combating predatory manatee hunting for illegal meat trade in the Americas.

KEY WORDS. Bushmeat; forensic markers; overhunting; SNPs.

The hunting of tropical wildlife has historically been conducted for subsistence consumption and for local trade. But current trends in wildlife harvest from across the globe suggest that the volume of extraction of wild game, or "bushmeat", has increased considerably, and many species are in sharp decline due to over exploitation (Albrechtsen *et al.* 2007, Bennett *et al.* 2007, Milner-Gulland *et al.* 2003, Redford 1992, Eaton *et al.* 2009). Species identification based on DNA analyses using biological material obtained from illegal hunting has been used in forensic genetics in European and African countries where hunting is prohibited. The identification of mitochondrial DNA polymorphisms is one of the most efficient methods of species differentiation.

Molecular markers such as single nucleotide polymorphisms (SNPs) can be detected using methods such as polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP), which are reliable, sensitive and inexpensive. These methods can be used for the detection of species-specific mutations, which enable classification of samples within a molecular taxonomy perspective (Morin *et al.* 2004, González *et al.* 2009). Variation within the cytochrome b gene has been found to be one of the most useful genetic markers for species discrimination (Meyer 1994, Palo & Merilä 2003, Riddle *et al.* 2003, Yan *et al.* 2005, Torres 2006, Parson *et al.* 2000). SNPs can be broadly defined as any single base substitution/indel in the genome of an individual (Primmer *et al.* 2002). The ability to identify wildlife products, whether as processed meat, skins or whole animals, is possible through by the development of DNA sequence databases using a standardized gene fragment (Ratnasingham & Hebert 2007, Ross *et al.* 2003, Eaton *et al.* 2009).

Given that the prospection of SNPs is still dependent of a prior knowledge of the species genome, Aitken *et al.* (2004) tested a method to determine the feasibility of using previously published comparative anchor tagged sequences (CATS) in order to find SNPs for mammal species with poorly known genomes. The authors demonstrated that, for most mammals, the targeted locus approach might provide an efficient and cost-effective method of discovering SNPs.

Sacks *et al*. (2009) established a SNP genotyping assay for detection of genetic variation in coding regions associated with coat-color alleles, phylogenetically basal mitochondrial mutations, sex markers, and non-coding parts of the fox genome. Malisa *et al*. (2006) described a protocol based on restriction enzyme digestion of PCR amplicons (PCR/RFLP) from a cytochrome b gene fragment that is useful in the identification of 13 wild African species directly affected by illegal hunting. DNA sequences have been used as complementary tools to construct phylogenies, determine population structure and identify species in different conservation programs directed at marine mammals (Baker & Palumbi 1994, Parsons *et al*. 2006, Wolf *et al*. 2007, Gillett *et al*. 2008).

Sirenia is an order of aquatic, strictly herbivorous mammals divided into two families: Trichechidae, with three species – *Trichechus manatus* Linnaeus, 1758, *Trichechus senegalensis* Link, 1795, *Trichechus inunguis* (Natterer, 1883) –; and Dugongidae, with one extant species – *Dugong dugon* (Müller, 1776) – and one extinct species – *Hydrodamalis gigas* (Zimmermann, 1780). The major cause of this extinction was extreme hunting in the 18th century (Berta *et al*. 2006). The distribution range of these species is restricted to warm waters in tropical, subtropical and equatorial areas (Vianna *et al*. 2006).

According to the Red List of the International Union for the Conservation of Nature (IUCN 2010), all manatee species are considered vulnerable (VU). Moreover, *T. manatus*, *T. inunguis* and *D. dugon* are listed in CITES (Convention of International Trade in Endangered Species of Wild Flora and Fauna) Appendix I and *T. senegalensis* are listed in CITES Appendix II (Cites 2010). Appendix I lists species that are the most endangered among CITES-listed animals and plants and Appendix II lists species that are not necessarily now threatened with extinction but that may become so unless trade is closely controlled.

The major causes of decline in Sirenia populations are accidental and intentional catches, collisions with boats and habitat loss (Lima *et al*. 2007). Both Brazilian manatees (*T. manatus* and *T.inunguis*) have been exploited by predatory hunting since the 16th century (Luna *et al*. 2008). Best (1982, 1984) estimates that between 1935 and 1954, 10,000 Amazon manatees (*T. inunguis*) were killed yearly.

In the northern Brazilian coast, marine manatees are still essentially captured for meat consumption (Luna *et al*. 2008). Due to the large sizes of the specimens, hunters also sell part of the meat in order to make some additional money. The leather of the manatees is used as medicine (poultice) in cuts and swelling, and as tea. The fat is used as medicine, as well as being used to maintain and fry the flesh of the manatee (Luna *et al*. 2008).

Currently, bioinformatics is essential for the manipulation of biological data, when combined with molecular biology tools allows the development of *in silico* studies involving genetic resources conservation, remodeling phylogenetic, assessment of gene dispersal and search of genomic markers (Rossi & Martella 2006).

The GenBank, one of the most complete and popular sequence databases, has more than 100 million nucleotide sequences from thousands of species. This enormous quantity of data offers an immense possibility for research and new discoveries. The *in silico* analysis is an obligatory first step to work with SNP's, mainly when the species studied is rare and endangered. Thus, the goal of the present study was to identify, *in silico*, nucleotide mutations in the cytochrome b gene capable to differentiate manatee meat from domestic species meat, commonly used as food sources. For such purposes, complete and partial Cytochrome b sequences from *T. manatus*, *T. inunguis*, *T. senegalensis*, and *D. dugon* were retrieved from *GenBank* in FASTA format. A local database was created using these manatee sequences and additional sequences from four domestic species, thereby allowing further comparisons (Tab. I).

Table I. List of species studied, with GenBank Access Number.

Species	GenBank Access Number
Bos indicus	EF061244
Ovis aries	DQ903227
	D84205
Capra hircus	EU350133
	D84201
Sus scrofa	AB376964
	AY830174
Trichechus manatus	AY965883
	AY965884
	AY965885
	AY965886
	D83050
Trichechus inunguis	AY965887
	AY965888
	AY965889
	AY965890
Trichechus senegalensis	AY965880
	AY965881
	AY965882
Dugong dugon	U07564
	AY075116

Nucleotide similarities between sequences were identified using the Sequencher 4.8 program (Gene Codes). Sequence alignments were performed with Clustal W of the BioEdit 6.0.7 pro-

gram (Hall 1999). Data mining of the alignments and minor adjustments were performed by eye. Nucleotide polymorphisms and restriction enzyme sites were found using the Cleaver software (Jarman 2006). After this procedure, we used the pDRAW 32 software v. 1.1.106 (Acaclone 2009) for selection of these enzymes and the generation of virtual gel electrophoresis.

The local database comprised five sequences obtained from *T. manatus*, four sequences from *T. inunguis* (615 bp) and seven complete sequences (1140 bp) from domestic animals. The alignments revealed five polymorphic sites distinguishing the manatee species from domestic species. Such SNPs were related to restriction enzyme digestion sites (Tab. II). Two additional sites were capable of differentiating the manatee species (light-gray sites in Tab. II).

A global analysis of the local database was carried out by adding three Cytochrome b sequences from *T. senegalensis* and two sequences from *D. dugon* (615 bp). The alignments revealed two polymorphic sites distinguishing all manatee species from domestic species. These SNPs were discriminated by restriction enzyme digestion with BanI and EcoRV (Tab. II). Two additional sites were capable of differentiating part of the manatee species as explained further in the text (light-gray sites in Tab. II). A total of four enzymes were identified as efficient in detecting seven diagnostic SNPs (Tab. II).

Analyzing the virtual gel electrophoresis it was observed that, by using the enzymes BanI and EcoRV, it is possible to distinguish between domestic species and manatees (Fig. 1). To discriminate *T. inunguis* from *T. manatus*, *T. senegalensis*, and *D. dugon* we used the enzyme HpyCH4III that yielded distinct RFLP profiles (Fig. 2). In addition, the enzyme MwoI also diagnosed the manatee species (Fig. 2). It is important to emphasize that this protocol is unable to differentiate between *T. manatus* and *T. senegalensis* at the cytochrome b gene.

The present study reinforced the potential use of cytochrome b polymorphisms as powerful markers for species identification, especially in vulnerably endangered species, such as manatees. The data provided herein should be useful as conservation tools for combating predatory manatee hunting for illegal meat trade.

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Table II. Sequence positions of SNPs and diagnostic restriction enzymes. (PS) Polymorphic site related to the cytochrome b complete sequence of *T. manatus* (NC_010302). Dark-gray sites are diagnostic SNPs between domestic species and manatee species. Light-gray sites are diagnostic SNPs between manatee species.

Enzymes	PS	*B. indicus*	*O. aries*	*C. hircus*	*S. scrofa*	*T. manatus*	*T. inunguis*	*T. senegalensis*	*D. dugon*
BsaHI	219	G	A	A	A	C	C	–	–
BssSI	606	A	A	A	A	G	G	–	–
XcmI	300	A	A	A	A	T	T	–	–
BanI	474	A	A	A	A	C	C	C	C
EcoRV[1]	243	C	A	C	C	C	C	C	T
	411	A	A	A	A	G	G	G	A
HpyCH4III[2]	367/432/580	G/N/A	A/N/A	A/N/A	G/N/G	A/N/G	A/N/A	A/N/G	G/N/A
MwoI[3]	259/580	G/A	G/A	G/A	G/G	G/G	G/A	G/G	G/A

[1] One restriction site in position 243 only in *D. dugon* and another restriction site in position 411 in *T. manatus*, *T. inunguis*, and *T. senegalensis*. [2] Three restriction sites for HpyCH4III, and the position 580 is useful on discriminating *T. manatus* and *T. senegalensis* from *T. inunguis* and *D. dugon*. N indicates any nucleotide. [3] Two restriction sites for MwoI and the position 580 is also useful on discriminating *T. manatus* and *T. senegalensis* from *T. inunguis* e *D. dugon*.

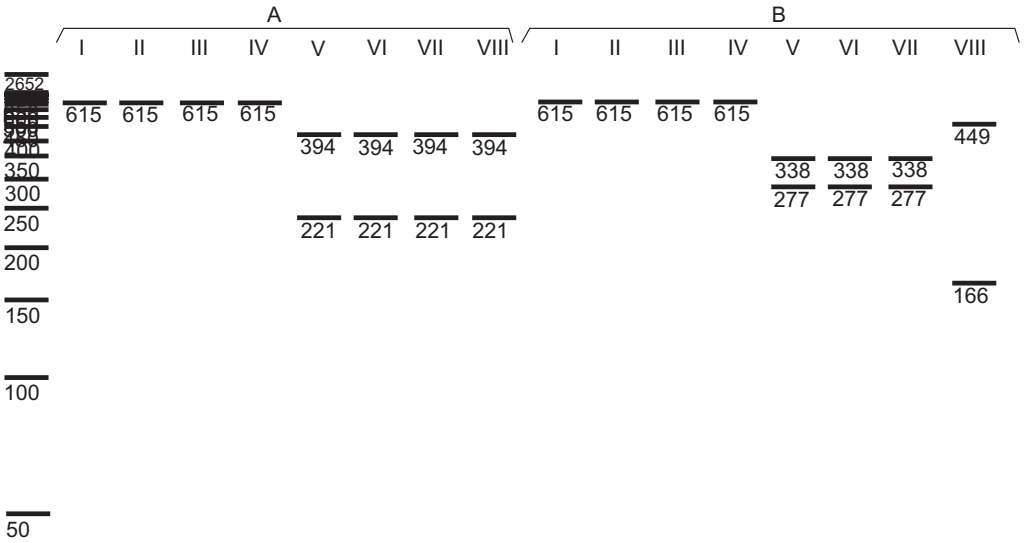

Figure 1. Virtual gel electrophoresis of Cytochrome b RFLP profiles for the four domestic species and for four manatee species under consideration. The Cytochrome b fragment is digested with BanI (394+221bp) (A), EcoRV (338+277bp or 449+166bp) (B) only in the *T. inunguis*, *T. manatus*, *T. senegalensis* and *D. dugon*. Identification of: (I) *Bos indicus* Linnaeus, 1758; (II) *Capra hircus* Linnaeus, 1758; (III) *Ovis aries* Linnaeus, 1758; (IV) *Sus scrofa* Linnaeus, 1758; (V) *T. inunguis*; (VI) *T. manatus*; (VII) *T. senegalensis*; (VIII) *D. dugon*.

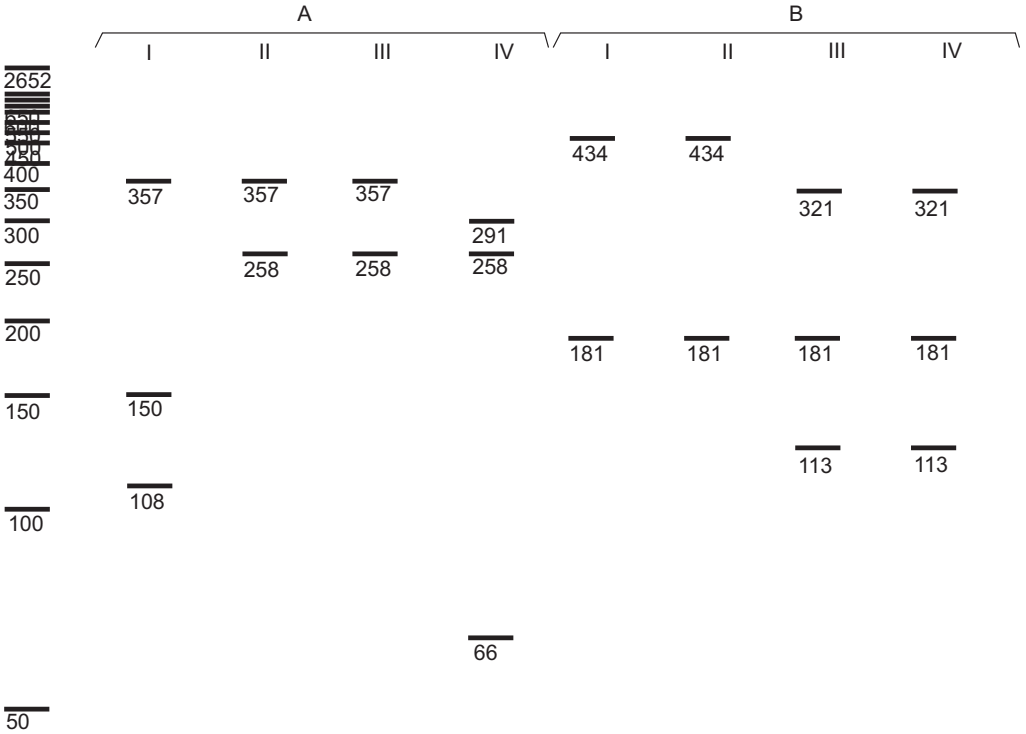

Figure 2. Virtual gel electrophoresis of Cytochrome b RFLP profiles for the four manatee species under consideration. The Cytochrome b fragment is digested with HpyCH4III (A) which yields a distinct RFLP profile of 357+150+108bp (*T. inunguis*) (I) ; 357+258bp (*T. manatus* and *T. senegalensis*) (II and III) ; 291+258+66 (*D. dugon*) (IV). Digestion with MwoI (B) yields distinct RFLP profiles of 434+181bp (*T. inunguis* and *D. dugon*) (I and II), and 321+181+113bp (*T. manatus* and *T. senegalensis*) (III and IV).

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